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RAW 264.7 mouse macrophage cells have interesting growth properties. To best utilize these cells in research protocols, rapid and inexpensive monitoring of in situ growth characteristics are necessary. An inexpensive computer imaging and visualization system was assembled to complement the microscopical analysis of in situ cells. Data for image analysis was provided by a combination of optically scanned cell cultures, framed-grabbed light microscopy, and optically scanned scanning and transmission micrographs of in situ cell cultures. The image analysis system employed included a Macintosh IIci CPU, frame grabber board, microscope, optical scanner, and software at a cost of less than $15,000. All but the transmission electron microscopy could be performed on the same cell sample with good results. The imaging system proved to be quite rapid. Several elements concerning RAW cell growth were uncovered including a relationship between cell culture seed concentration and the appearance of foci. However, the mechanism of viral expression is still unclear. The imaging system was successfully adapted for looking at bacterial cultures as well. A low-cost imaging system is now in place for examining in situ cell culture systems of several types.

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IMAGE ANALYSIS OF VIRAL-EXPRESSING MOUSE MACROPHAGE CELLS

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Introduction

History

This research effort was undertaken to take advantage of initial results obtained during a cooperative research program between Dr. Johnathan Kiel of the Directed Energy Division at Brooks Air Force Base and Robert V. Blystone of the Department of Biology at Trinity University. Dr. Kiel has an ongoing program investigating various effects of radiofrequency radiation on cells. Dr. Blystone has had two AFOSR summer faculty fellowships (1989 and 1990) to perform research at Brooks Air Force Base. Dr. Kiel's research required morphological investigation of cells he was using in ongoing research protocols. One cell line was of particular interest: RAW 264.7 mouse macrophage cells. Dr. Blystone's background in microscopy was brought to bear on the RAW cell investigation. During that investigation, it was discovered that RAW cells could produce virus. This finding suggested additional investigation. The report here describes the additional effort to better understand the RAW cell line as it is being used in Dr. Kiel's laboratory.

Objectives of Study

The initial objectives stated in the proposal were as follows:

1. to better understand certain properties of the RAW 264.7 mouse macrophage cell line by means of computer image analysis of micrographic data.

2. to develop a model for an inexpensive image analysis system that can correlate morphological data with biochemical / molecular data.

This project has been extremely successful in accomplishing the second objective of developing an inexpensive image analysis system. The first objective has also been met although the focus of the RAW cell properties to be studied changed during the course of the investigation. Instead of following the viral production of RAW cells, growth dynamics and foci formation of RAW 264.7 cells became the more profitable focus of research.

The RAW cell model

The RAW 264.7 cell line was established in 1978 (Raschke et al., 1978). This macrophage tumor was the result of a transformation produced by Abelson's Leukemia Virus. Upon culture the cell line will avidly produce foci. Additionally, the line will secrete Tumor Necrosis Factor (TNF). The ability to produce TNF can be used as a marker of the physiological state of the cell. It is this quality of RAW 264.7 that suggests its use in various research protocols.

RAW 264.7 had been selected as a cell line to monitor in RF fields. Changes in TNF production would indicate response to microwave application. Although the biochemical response to microwave treatment had been followed, the possible morphological response of the cell line was unknown. This macrophage has been poorly characterized in terms of fine structure.

The initial microscopy study revealed the presence of C-type virus in the cell cultures. The report by Raschke et al. (1978) indicated that RAW 264.7 cells did not easily express virus. Further, the presence of virus could alter the interpretation of earlier experimental results as it introduced another variable into the experiments. The RAW macrophage as a cell model needed further consideration.

The initial transmission electron microscopy of RAW 264.7 utilized an in situ approach to view the cells. Cultures were established on 8-well Lab-Tek 1 by 3 inch chamber slides. They were fixed in place, embedded in plastic, and then released from the culture glass. Transmission electron

microscopy (TEM) suggested that the viral particles were released from specific sites while the cells were in the confluent monolayer state. New developments in image analysis offer possible methods for visualizing the viral release from RAW cells.

This research was, therefore, stimulated by a need to better understand the viral expressing ability of RAW cells and to employ new image analysis methodology to follow the process.

Materials and Methods

Initial Methods considerations

RAW 264.7 cells were cultured in RPMI 1640 medium with 10% fetal bovine serum added. In a 37°C environment with 5% CO_2, the cells grow vigorously. The doubling times are from 18 to 24 hours. With a typical seeding, a T-75 culture flask needs to be split every seven days or so.

Microscopy (light and electron) revealed that the RAW cell lawn on the Lab-Tek well slides was uneven. This uneven growth was obviated by the preparation technique for microscopy. Fixation was with 3% glutaraldehyde in 0.05 M phosphate buffer which was placed in each well of the culture slide. After one hour the fixative was removed and each well rinsed with buffer. Staining was accomplished with 1% OsO_4 in 0.05 M phosphate buffer and applied to the cells for exactly one hour. Because density measurements were to be taken of the cells, OsO_4 must be applied with great care. The sample was dehydrated in a graded ethanol series. The plastic well assembly and its gasket were removed at this time. Further cell preparation varied depending on the type of microscopy to be employed.

The glass slide would clearly picture stained squares of cells conforming to the outline of the plastic well assembly. Both visual and microscopical inspection revealed the cell lawn on the slide was uneven. The pattern of unevenness and the density of stain would vary with the age of the culture. These observations were made at about the time this proposal was funded and corresponded with the research period of the summer of 1990.

Growth characteristics

An early research consideration was the influence of the age of the culture on possible virus production. The age of a culture is influenced by the concentration of cells at which the culture is seeded. Heavily seeded cultures would reach monolayer confluence earlier and leave log phase growth sooner. Further, older cultures are more sticky due to the presence of accumulated extracellular matrix. A series of experiments was established to follow the effect of seed size and age on RAW cell growth.

The maximum volume of an 8-chamber Lab-Tek well is about 0.9 ml. An appropriate seed volume is 0.5 ml for this size well. It should be noted that the well dimensions produce a pronounced meniscus effect. This effect will be referred to later.

Cells were cultured in T-75 flasks in two ways: 1) cells were allowed to grow into dense monolayers with many foci and 2) cells were allowed to grow only to the point where they were almost confluent monolayers. Condition one represented old cells and condition two represented young log-phase cells.

Cells from old and new growth states were seeded into Lab-Tek chambers at seed values of 10,000, 20,000, 50,000, 100,000, 200,000, and 500,000 cells per ml. The cell seeds were prepared in the following fashion. The old media was poured from the T-75 flask. 10 ml of phosphate buffered saline was added. The flask surface was scrapped of cells. The released cells were poured off. The flask was rinsed once with 10 ml phosphate buffered saline and the rinse added to the original cells. The cells were gently pelleted by centrifugation. The pellet was resuspended and washed with 10 ml of phosphate buffered saline. The cells were again gently pelleted. Then the RAW cells were resuspended in from 1 to 5 ml of RPMI media. The cell density was determined with either a Coulter Counter calibrated for macrophages or by means of a hemacytometer. Samples of the appropriate concentration were diluted with RPMI media. Then cells of different concentrations were seeded to the culture wells. The different cell density and age combinations were incubated for varying times (1,2,3,4 and 5 days).

The cells were then prepared by microscopy by the glutaraldehyde/osmium procedure described earlier.

Cells seeded at high concentrations and cultured for long times showed considerable overgrowth and foci formation. Cells seeded at low concentrations demonstrated very irregular distribution patterns on the culture well surface. A decision was made to concentrate on the growth dynamics and lay aside the virus production investigation. This decision was further reinforced by cell imaging developments.

Cell imaging and visualization equipment

The following computer equipment was assembled for this study. The grant provided a Macintosh IIci operating under AUX-2. The CPU was fitted with 20 megabytes of RAM, an internal 80 megabyte hard drive, an external 600 megabyte hard drive, an ethernet board, and a RasterOps 264 color card. Provided by other sources was a Macintosh IIci with 8 megabytes of RAM, an internal 80 megabyte hard drive, an external 105 megabyte hard drive, a Data Translation DT-2255 frame grabber board, a Zeiss BK microscope with Plan objectives and a Javelin 2/3 inch MOS RGB chip camera. A third computer used was a Macintosh SE30 with 5 megabytes of RAM, an internal 40 megabyte hard drive, a RasterOps 364SE color card, and an attached HP ScanJet Plus optical scanner. All CPUs had 13 inch Apple RGB high resolution monitors. Software packages that were available included the following: MacRenderman, StrataVision, Spyglass, NCSA Datascope and Dataslice, X-windows, MacX, NIH Image, Enhance, Photoshop, Statview, JMP, CricketGraph, Deltagraph, Deskscan, Excel, Word, and MacPaint. All three CPU's were networked through Local Talk and file transfer was possible with Okyto software.

One computer could perform most of the tasks described below. This configuration would consist of a Macintosh IIci, 8 megabytes of RAM, 80 megabyte hard drive, color monitor, Data Translation DT-2255 frame grabber board, HP ScanJet Plus optical scanner, a light microscope, Javelin RGB camera, NIH Image software, CricketGraph, and Word. This minimal system would cost well under $15,000 to assemble.

It must be appreciated how long it takes to be conversant with so many software packages and with two CPU operating systems: AUX-2 and Mac-OS. At one time Sun microcomputers were envisioned as being necessary for this project; however, AUX-2 on Macintosh unobviated the need for Sun workstations.

With the computer components described above, the following image manipulations are possible.

1. Direct optical scanning of unstained 8-well Lab-Tek culture slides.
2. Direct optical scanning of stained culture slides.
3. Digital capture of light microscope images.
4. Digital capture of optically scanned electron micrographs.
5. Creation of digitally replayed movie sequences from serial image capture.
6. Image enhancement and coloring of any digital image.
7. Automated counting of structures within a digital image.
8. Production of histograms of gray scale distributions.
9. Optical density recording based on gray scale values.
10. Thresholding and kernaling of digital images.
11. Sizing and scaling of digital images.
12. Morphometry.
13. Animation of digital images.
14. 3-D rendering and rotation of digital images.
15. Isosurface mapping of digital images.

These and other imaging and visualization routines are possible even with the minimal equipment configuration described above.

<u>The experimental procedure</u>

Given the equipment and the initial growth experiments, it was decided to develop the following procedure for the study of RAW cells. Much of the time spent during the grant period was directed to the development and implementation of the six steps below.

1. Seed log phase harvested cells at the rate of 100,000 cells/ml.
2. At intervals of 1, 2, 3, 4, and 5 days incubation prepare wells for microscopy.
3. Optically scan the stained cells on their culture slide directly.

4. Examine the same cells in the light microscope.
5. Prepare the same cells for scanning microscopy and observe in the SEM.
6. Again with the same cells, prepare specimen for transmission electron microscopy and observe in the TEM.

This protocol would allow the observation of in situ, vigorous cells with four levels of computer based visualization at five distinct time points. A discussion of each step of the protocol follows.

Step 1. A seed rate of 100,000 cells/ml of log phase harvested cells gave the most even distribution of cells over the surface of the culture well for the growth intervals envisioned.

Step 2. Cells required about one hour to adhere to the well surface. At 24 hours the first doubling of cells was usually complete. Assuming cell doubling every 24 hours, the culture surface would be statistically covered after about four days incubation.

Step 3. The optical scanner is an 8 bit, 256 gray scale machine. A 1 by 3 inch slide requires approximately 300K of memory to hold a digitized image at the highest machine resolution. This resolution results in gray scale increases of density of from between 10 and 20 units per day of culture, well within the range of the optical scanner. This represents another reason for choosing the 100,000 cell/ml seed concentration. Examination of image processed optical scans can suggest areas of potential interest for microscopy.

Step 4. Light microscopy can be brought to bear on special areas of interest within a well. One problem did arise: the microscope objectives were calibrated for 0.17cm working distance. The specimen slide could not be used with a cover slip because of subsequent handling. So the resolution of the light microscope was not the best. Different working distance objectives could improve the resolution. Light microscopy was especially useful for identifying foci. The software employed could perform automated foci counts although improvement is possible. The system can not discriminate foci volume well, however.

Step 5. After digital light microscopy, the cells were coated with gold and taken to the SEM. Scanning electron microscopy proved to be the most valuable tool in visualizing the cells. The videotape which accompanies this report clearly demonstrates how computer visualized SEM reveals a great deal of information about RAW cells and their foci interactions. This technique and optical scanning of well slides proved to be the most valuable techniques developed by this study. Accurate cell measurements were possible with SEM, far better than by light microscopy.

Step 6. After SEM, plastic-filled Beem capsules were inverted over the cells. The plastic was polymerized and the glass-cell-plastic combination was chilled. The plastic blocks were snapped off of the glass, bringing the cells along with the plastic. The cells now embedded in plastic were sectioned and examined in the transmission electron microscope. The results obtained with these "rescued for TEM cells" was quite poor. An extension of the grant period was sought and given in order to work out this technique. Improvement in image quality was not to be. The handling of the cells for the other imaging techniques deteriorates the cells in so far as TEM imaging is concerned. It is not possible to do *in situ*, same-cell imaging with TEM. TEM is important because it is the best way to visualize virus particles.

To summarize, the methodology employed to visualize the growth dynamics of RAW cells is outlined in the six step process listed above. The majority of the time spent on this study was used in developing the computer methodology for analysis and scientific visualization of the cells.

Results

Foci formation and related issues

Two elements of RAW cell growth gathered much attention: evenness of growth and foci formation. Culture wells were observed in the inverted microscope immediately after seeding and followed periodically over the course of the first hour. Cells for new cultures derived from cell passages

that had been maintained in culture for a long time tend to stick together. The cells settle to the new culture well bottoms in multiples. Cells for new cultures that are derived from cell passages that are in log phase growth are far more likely to settle as single cells. These latter cells have a more random distribution. Old source cells are more likely to form foci early in new cell culture and have non-random distribution patterns.

Foci forming is an important tool in determining the transformation of cells induced by biological agents and chemicals. Many techniques rely on quantitating foci formation for up to 30 days after seeding a culture. At the suggestion of Dr. Kiel, an attempt was made to determine if the computer system could automate foci counting and perform counting early in cell culture. Thus, it was quite important to understand the early stages of foci formation by a cell line that is very good at forming foci, RAW cells. It is clear that the age of the initial culture cells can influence early foci number.

Foci counting is a relatively simple process using NIH Image. The cluster of cells are manipulated to reduce non-cell areas between cells in a cluster; the imaging processes used are called smoothing and eroding. The cells are then thresholded; this means that all gray scale values below certain levels are set to zero. The image is then binarized; this means all image data is either rendered black or white. The software is then instructed to count all objects in either the white or black field. Foci can be quickly counted in this fashion. A difficulty does arise. The system does not know how to account for depth and, thus, volume. Some foci have more vertical elevation than horizontal. This means that diameter discrimination for size differences in foci are not reliable. The technique is promising and requires more investigation.

<u>The uneven distribution problem</u>

The cell lawns on the glass surface often favored the edge of the wells in terms of growth or at least staining density. And the middle cell wells seem to support growth better than the outer cell wells. These observations need careful examination. The meniscus of growth media seen in the well chamber may exert pressure gradients or diffusion gradients

that favor perimeter growth. Time did not allow this problem to be adequately followed although experiments are now underway (after the grant period was over) using different well configurations.

Optical density measurements

One of the most promising outcomes of the computer visualization methodology development was measuring cell growth with a flatbed optical scanner. With eight wells on a Lab-Tek slide, multiple experiments can be easily and inexpensively established. One well was left as a blank which would contain only growth media. Any staining response from this chamber would be subtracted as background noise from the other chambers. Individual wells can be examined with Enhance software and the average gray scale density over the whole surface of one well determined with standard deviation calculations possible. With NIH Image the gray scale values for each point on the well can be set into a three dimensional gray scale map. Currently, these maps are being rendered and rotated in three dimensional space so that the surface topography as represented by gray scale values can be more clearly seen.

3-D reconstruction

A primary goal of this work was to reconstruct a three dimensional view of RAW cells based on serial TEM work. Quality TEM photomicrographs could not be produced fast enough to supply material needed for the development of the technique for 3-D reconstruction. Serial sections of embryonic pig and chick were substituted to provide digital data sets needed to develop the 3-D rendering techniques on low cost CPU platforms. The enclosed video provides some evidence of a movie of the serial 2-D pig embryo sections being used in the reconstruction experiments. Work is continuing in this area.

Discussion

New Developments

During the grant research period three developments took place at Brooks to which attention was paid. These developments were not part of the original

proposal. First, Dr. Kiel's laboratory isolated a growth factor from the RAW cells. The computer methodology is being modified to work on this finding. In particular, unstained living RAW cells are now being optically scanned and the same culture wells are being followed over time as opposed to reconstructing growth from multiple stained wells. This could be an exciting outcome. In a demonstration project, culture wells of different incubation ages were splined together and run as a movie. The result, after pseudocoloring, was like watching the movement of a rain storm over the surface of the earth. The cells could be visualized growing and moving based on density changes.

Secondly, Dr. Kiel is investigating bacterial response to several microwave potentiating chemicals. Of particular interest is how the bacteria can tie themselves into knots. By modifying the optical scanning procedures, the bacteria, while still on their petri plates, were scanned. Colony differences based on media differences could be very quickly quantitated. The treated bacteria were also examined by electron microscopy and preliminary results indicated that the bacteria changed significantly in their diameters, an unexpected result.

Third, Dr. Frei has collected a vast array of data representing thermal responses by rats to microwave radiation. Dr. Frei is seeking a way to depict this very information dense data in a more simple visual approach. The videotape accompanying this report shows an early effort to visualize thermal-like data. A model of a head was digitized and then a pseudocolor thermal map based on grayscale values was mapped to it. Dr. Frei's data could be handled in a similar fashion and mapped to a rotating rat.

In conclusion, a methodology has been developed to visualize cells. The methodology is highly adaptable and inexpensive. The presence and availability of the methodology and equipment has become a resource to several investigators at Brooks Air Force Base. Also RAW cell growth has been better characterized in the 8-well environment. The experience gained in computer visualization is now being put to several new applications.

Additional Outcomes

Resources provided by the AFOSR grant have had many positive outcomes. In conjunction with an AFOSR-UES Summer Faculty Fellowship minigrant, these additional benefits were accrued.

1. A research presentation was given to the International Congress for Electron Microscopy.

   R.V. Blystone, J. Kiel, J. Parker, and C. Collumb. 1990. Expression of viral particles by RAW 264.7 mouse macrophage cells after treatment with lipopolysaccharide. Proceedings of the XIIth International Congress for Electron Microscopy, Volume 3 - Advances in Microscopy in the Biological Sciences: 574-575.

2. A research presentation was made at the American Society for Cell Biology annual meeting.

   R. Blystone, C. Collumb, T. Romo, and J. Kiel. 1990. Low-cost image analysis of <u>in situ</u> mouse macrophage cell growth characteristics. Journal of Cell Biology 111 (5 part 2): 57a.

3. Two manuscripts are in preparation. One paper deals with low-cost scientific visualization and the other with RAW cell growth.

4. Two research presentations are in preparation. One presentation reports on the bacterial measurement with the outlined methodology and the second with computer animation of cell growth dynamics

5. Two undergraduate students were employed with grant resources. One has been accepted into the Computational Biology graduate program at Rice University. The other has been accepted into the Marine Biology graduate program at William and Mary College.

6. Experienced gained in computer assisted microscopy provided by the grant contributed to NSF-ILI funding for a $95,000 undergraduate biology computer visualization teaching laboratory.

7. Activity in the laboratory warranted the American Society for Cell Biology funding a stipend for a high school biology teacher to work in the computer lab during the summer of 1991.

8. Continued collaboration with Dr. Kiel and Dr. Frei.